

Harvest time and ensilage suitability of giant reed and miscanthus for bio-methane production and characterization of digestate for agronomic use

F. da Borso, C. Di Marzo, F. Zuliani, F. Danuso and M. Baldini*

Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, Via delle Scienze, 206, IT33100 Udine, Italy

*Correspondence: mario.baldini@uniud.it

Abstract. In many countries, biogas plants are mainly fed by livestock slurry and dedicated crops, including maize, which still represents one of the main energy crops utilized. Many concerns are now arising on environmental impact due to the high water consumption, chemical fertilizer and pesticide requirements and on adverse effect of maize as energy crop on the price of food and feed commodities. For these reasons two perennial crops, in particular miscanthus (*Miscanthus x giganteus*) and giant reed (*Arundo donax* L.), were cultivated at very low input and evaluated for their bio-methane yield at different harvest times and ensilage suitability, in a north-eastern area of Italy. Moreover, considering the agronomic use of the obtained digestate as fertilizer, this has been characterized by the content of heavy metals. Both multi-annual crops have proved highly productive in biomass especially with a harvest time in autumn, at which a satisfactory completion of the silage process without additives was observed. Conversely, bio-methane yield per hectare were not satisfactory with respect to the reference crops such as maize. The low BMP attained showed the main bottleneck of the methanisation of ensiled giant-reed and miscanthus, which is represented by fiber composition with high degree of lignification. The simulation use of digestate obtained as fertilizer in vulnerable areas, could lead to slightly exceed the levels allowed by the legislation of some European countries with regard of heavy metals as Cu, Zn and Cd.

Key words: multiannual crops, biomass, silage, energy, bio-methane, digestate.

INTRODUCTION

The agricultural sector must participate in the effort in promoting the conversion from a fossil fuel-based to a bio-based economy (Richardson, 2012), supplying biomass to be transformed into various forms of energy. Among them, biofuels including methane represent, an important strategy to reduce GHG, thus complying with the Kyoto Protocol and subsequent legislation. In Europe, maize is the most commonly used energy crop as biogas feedstock, especially in Germany (Weiland, 2006) and Italy (Carrosio, 2013), the two main biogas producing countries in the European Union. However, the cultivation in fertile agricultural land with high input crop-management techniques made the maize, as energy crop, responsible of elevated environmental impact, increment in food price volatility and in associated risks for food security (FAO, 2008). The use of multi-annual

species, in particular miscanthus (*Miscanthus x giganteus*) and giant reed (*Arundo donax*), resulted in clearly positive environmental loads and able to valorising marginal land (Hastings et al., 2008; Fazio & Monti, 2011; Cadoux et al., 2014), could overcome these drawbacks. Although both crops are subject of several recent anaerobic digestion (AD) experiments, especially in Mediterranean area (Lewandoski et al., 2003; Heaton et al., 2004; Angelini et al., 2005; Angelini et al., 2009; Mantineo et al., 2009; Massé et al., 2010; Ragaglini et al., 2014), the amount and quality production and the specific species to adopt remains strictly affected by different climate conditions and management practices of the specific environment (Beale & Long, 1997; Heaton et al., 2009; Arundale et al., 2014). In particular, harvest time is a major factor determining biomass productivity (Beale & Long, 1997; Heaton et al., 2009; Hoagland et al., 2013), quality (Kludze et al., 2013; Baxter et al., 2014) and, consequently, the efficiency of the biochemical processes affected by fibre composition in microbial activity during fermentation (Klimiuk et al., 2010; Monlau et al., 2013). Although ensilage is today commonly performed in maize and other grasses to preserve biomass until use for AD in many farm biogas plants (Yahaya et al., 2001; Neureiter et al., 2005; Vervaeren, et al., 2010; Herrmann et al., 2011), studies on naturally occurring ensilage of giant reed and miscanthus, with subsequent silage utilization in an AD experiment for methane production, are still limited (Dragoni et al., 2015; Liu et al., 2015).

A complete study on the efficiency and environmental impact aspects on giant reed and miscanthus biogas chain cannot be limited to the cultivation aspect of both crops, but also the chemical characteristics of the main AD co-product, such as the digestate, generally used as fertilizer in agricultural practices, could be of great interest. The EU Nitrate Directive (Council Directive 91/676/EEC and followings) states that agricultural use of livestock manure must comply with the limit of 170 kg N ha⁻¹ and per year in vulnerable zones; consequently the knowledge of nitrogen content is essential to estimate the distributable volume of digestate containing animal originated nitrogen. Moreover, heavy metal content in digestate or manure sludge represents another important aspect in using digestate as fertilizer; however, Italian law does not set limits for such products. In effect, the digestate if not properly checked for these pollutants elements, could contaminate susceptible soils, with great difficulty to remedy.

For the above reasons, we addressed the effects of harvesting time (summer vs. autumn vs. winter) on quantitative and qualitative biomass productions, ensilage suitability and biochemical AD process for bio-methane yield of giant reed and miscanthus silages in a northeast Mediterranean area. Furthermore, despite the digestate characterization is normally performed in real scale anaerobic digesters, operating under stable and continuous load conditions, nowadays these kind of data are lacking for giant reed and miscanthus and first approaches to their determination are needed. Consequently, the digestate of the both multiannual crops at the end of AD was submitted to a chemical evaluation in order to monitoring its nitrogen and heavy metals content, as the same will be used as fertilizer to distribute in the soils.

MATERIALS AND METHODS

Field experiment and biomass samples preparation

The field experiment was conducted at the Experimental Farm of Udine University, Udine, Italy (46° 04' N, 13° 22' E, height 109 m a.s.l. and 0% slope). The experimental site is characterized by a shallow soil (about 50 cm) and by a continental climate with main traits reported in Table 1 and Fig. 1, respectively.

In 2014, five years giant reed and miscanthus crops were compared for biomass and bio-methane yield, ensilage suitability and digestate characteristics as fertilizer. Harvesting was done on August (summer), October (fall) and December (winter) for both multiannual crops, in order to assess the influence of growth stage and meteorological conditions on biomass characteristics and methane yield.

The crop management details adopted for the experiment were reported in Baldini et al. (2017).

The experiment was organized following a split plot design with four replication, in order to analyse species (giant reed and miscanthus) x harvest time (summer, fall and winter) effects, with 8 main plots of 60 m² each (species), subdivided in 24 sub-plots (harvest time) of 20 m² each.

At harvest time, fresh and dry biomass (moisture content was obtained maintaining representative samples of fresh biomass at 105 °C until constant weight) was determined by hand sampling 6 m² area within each plot, discarding the border rows. On a subsample of ten plants per plot, number of green and lost leaves per plant were also determined. Representative samples were air dried in greenhouse conditions (30–40 °C), and stored at -18 °C for subsequent chemical analyses.

Ensilage lab experiment

At each harvest time, four plants were randomly chosen in each replication, chopped and placed in 1,000 mL sealed waterproof vessels, for a laboratory ensilage experiment, following the methodology already described by (Baldini et al., 2017). Plants harvested at winter harvest time, due to high dry matter content, prevented air excluding and a suitable packing for being submitted to ensilage process. Therefore, water was added to increase the moisture level before silage. 28 plastic vessels were used for the experiment, which, at the end of about 40 days, were stored at -18 °C prior to chemical analysis.

Biochemical Methane Potential (BMP) assay

To determine Biochemical Methane Potential (BMP), the Automatic Methane Potential Test System (AMPTS I, Bioprocess ControlTM, Sweden) was used. Incubation bottles of 400 mL containing triplicated samples of giant reed, miscanthus and inoculum were incubated at a temperature of 37 ± 1 °C, with an inoculum to substrate VS ratio (I:S) of 2:1 (Baldini et al., 2017). BMP of substrates was calculated net of methane production from inoculum as the cumulative methane yield at the end of the test and the relative VS content (NmL CH₄ g⁻¹ VS). Methane yields per hectare (Nm³ CH₄ ha⁻¹ year⁻¹) were calculated by the product of the organic substance produced taking into account of the ensilage losses (kg VS ha⁻¹ year⁻¹) and the relative BMP obtained (calculated as Nm³ kg⁻¹ VS).

At the end of anaerobic digestion tests, which lasted 36 days, the representative samples of digestate from each bottle were taken and stored at -18 °C for subsequent chemical analyses.

Chemical analysis

Representative subsamples were dried at 100 °C for 16 h in a fan-assisted oven to estimate total solids content (TS). The rest of the samples were dried at 50 °C for 48 h before being milled in a rotor mill equipped with a 1 mm grid for further qualitative analysis.

On representative samples of biomass, silage and digestate after AD, the content of oxygen, nitrogen, hydrogen and carbon was determined using an Elemental Analyzer (vario Micro cube, Hanau, Germany) operating on the principle of catalytic combustion under oxygen supply and high temperatures. Crude protein content (Dumas method) was calculated as 6.25 (conversion factor) multiplied by the elemental nitrogen content detected. Ether extract (EE) was determined according to Soxhlet method. Volatile Solids (VS) concentration was subsequently determined ($VS = TS - \text{ash}$).

Starch was calculated following the amyloglucosidase- α -amylase method (McCleary et al., 1997).

Hemicellulose, cellulose, lignin and non-soluble ash contents were determined on these dried and finely powered samples in accordance with the methods of Goering & van Soest (1970). Neutral detergent fiber (NDF) was first determined as the material remaining after heating with neutral reagent. Next acid detergent fiber (ADF) was determined as the material remaining after heating with a 0.5 M H₂SO₄-reagent for 1 h. Acid lignin fiber (ALF) was then determined as the material remaining after treatment with 72% H₂SO₄ for 24 h. Lastly, the non-soluble ash content was determined as the material remaining after combustion at 525 °C for 3 h in a muffle furnace. Hemicellulose was calculated as the difference between NDF and ADF, cellulose as the difference between ADF and ALF and lignin as the difference between ALF and non-soluble ash.

Soluble carbohydrates (SC), volatile fatty acids (VFA) and ammonia nitrogen (TAN) were obtained following the methodologies already described in Baldini et al. (2017).

The mineral elements and heavy metals contents were detected on digestate, in order to consider its further utilization as fertilizer in the field. Samples of digestate were oven-dried (105 °C for 48 h) and digested in 10 mL of 65% (v/v) HNO₃ in Teflon cylinders for 10 min at 175 °C in a microwave (CEM MARS). After digestion, samples were diluted to 20 mL with milliQ water, filtered through 0.45 μm filters pte and diluted 1:10 prior analysis with an ICP-OES (Vista MPX, Varian Inc.). The accuracy of the analytical procedure was checked running standards every 20 samples and quality control was conducted using Y (Yttrium) as the internal standard, reagent blank samples, and triplicates reading for each sample (USEPA, 2007). The main elements detected were: nitrogen (N), aluminium (Al), calcium (Ca), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), sodium (Na), manganese (Mn), nickel (Ni), phosphorus (P), lead (Pb) and zinc (Zn).

Statistical analysis

All data were subjected to two-way analysis of variance (ANOVA). A fixed-model was adopted, with species and harvest times as independent variables. When ANOVA revealed significant differences between means, *Student–Newman–Keuls* test at $P \leq 0.05$ was adopted to separate means. The term ‘significant’ is only used where a statistical analysis of significance has been performed. Means values of energy yield and digestate mineral elements are given ± 1 SE.

RESULTS AND DISCUSSIONS

ANOVA results and biomass yield

Table 1. Main soil characteristics (0–0.5 m layer)

Parameter	Unit	Value	Method adopted
Sand ($> 0.05 < 2$ mm)	%	43	Ministero per le Politiche Agricole (1999)
Loam ($> 0.002 < 0.05$ mm)	%	40	Ministero per le Politiche Agricole (1999)
Clay (< 0.002 mm)	%	17	Ministero per le Politiche Agricole (1999)
pH	-	7.35	In water solution; Ministero per le Politiche Agricole (1999)
Total calcareous	%	5.5	Gas-volumetric; Ministero per le Politiche Agricole (1999)
Active calcium carbonate	%	0.2	Drouienau; Ministero per le Politiche Agricole (1999)
Organic matter	%	1.8	Walkley and Black; Ministero per le Politiche Agricole (1999)
Total nitrogen	g kg ⁻¹	1.85	Kjeldahl; Ministero per le Politiche Agricole (1999)
C/N	-	10	-
Phosphorus available	mg kg ⁻¹	34	Ferrari; (AOAC, 1990)
Potassium available	mg kg ⁻¹	164	Dirks and Scheffer; (AOAC, 1990)
Cationic exchange capacity	Meq 100 g ⁻¹	18.2	Barium chloride; Ministero per le Politiche Agricole (1999)

The growing season 2014 (April–December) was characterized by an average minimum and maximum daily air temperature of 11.4 and 21.9 °C, respectively; maximum air temperature never peaked above 30 °C and rainfall amounted to 1,346 mm. The climatic conditions (Fig. 1) were quite similar to these recorded in the last 30 years, with the exception of a significant warmer fall–winter period (October–December, +2.0 °C as average temperature) and a slightly more dry springtime (April–June period, -50 mm of rainfall) (ARPA FVG–OSMER, 2016).

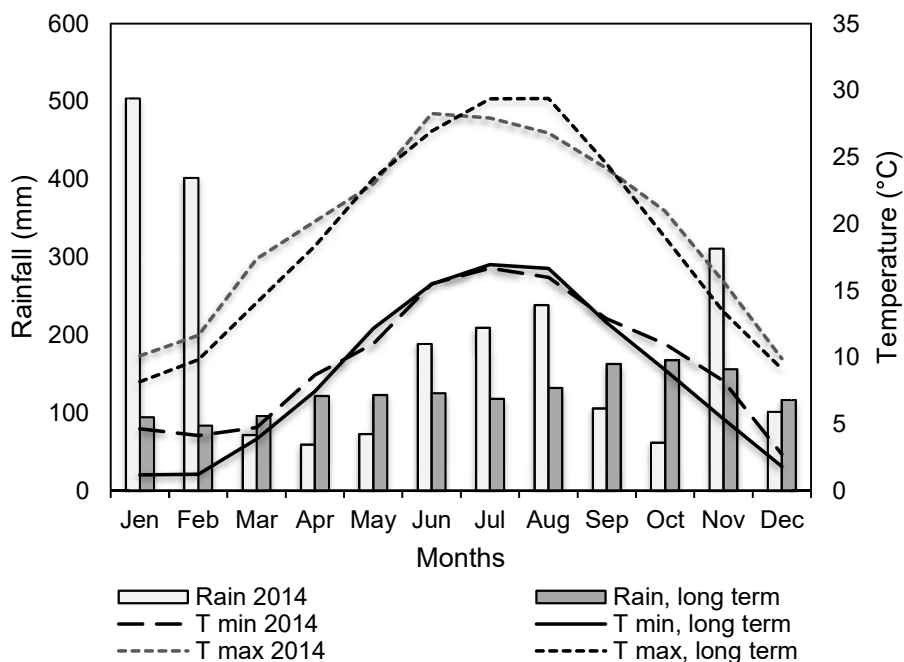


Figure 1. Monthly rainfall and average daily maximum and minimum air temperature patterns in the 2014 as compared to the long-term (20 years) average.

In Tables 2 and 3 are reported the ANOVA results of different traits.

Table 2. ANOVA results, biomass yield and qualitative traits of fresh biomass

Factors	biomass yield	dry matter	starch	sc	hem	cell	lign	prot
Harvest time	**	**	**	**	**	*	*	**
Species	**	**	**	**	n.s.	*	n.s.	*
Harvest time x species	x**	**	**	**	**	n.s.	n.s.	n.s.

*, ** – $P \leq 0.05$ and 0.01 , respectively; sc – soluble carbohydrates; hem – hemicellulose; cell – cellulose; lig – lignin; prot – crude protein.

Table 3. ANOVA results, qualitative traits of silage

Factors	starch	sc	hem	cell	lign	prot	lac	ac	pr	but	an
Harvest time	**	**	n.s.	n.s.	*	**	**	**	**	**	**
Species	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	**	**	**
Harvest time x species	*	*	n.s.	n.s.	n.s.	n.s.	**	**	**	**	**

*, ** – $P \leq 0.05$ and 0.01 , respectively; lac – lactic acid; ac – acetic acid; pr – propionic acid; but – butyric + isobutyric acid; an – ammonia nitrogen.

A significant increase in biomass yield was registered between summer and fall harvest for both crops (increase of 21.5 and 33.5% for giant reed and miscanthus, respectively), due essentially to the increase in stem height and leaves number (data not shown), with a negligible leaf loss (Fig. 2).

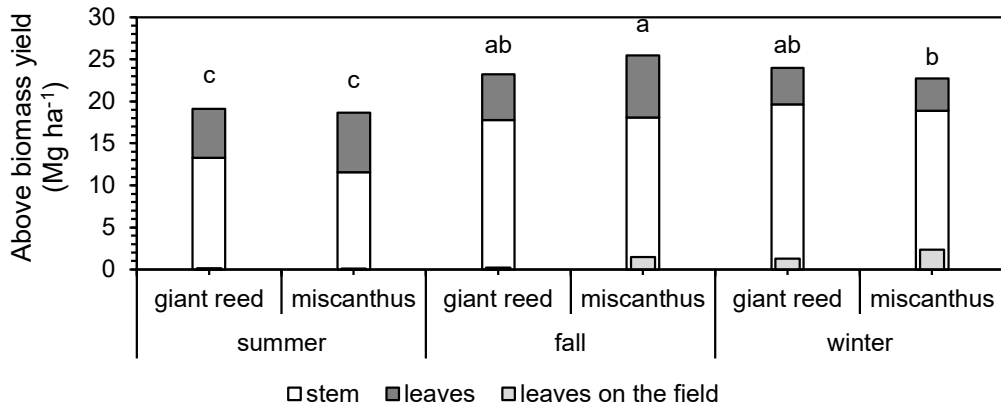


Figure 2. Partitioning of above biomass yield and lost production from fallen leaves on the field of giant reed and miscanthus at three harvest times (summer, fall and winter). For total above biomass yield, different letters indicate statistically different means (*SNK test*; $P \leq 0.05$).

The results obtained in the experiments, (Fig. 2), evidenced that the highest above dry biomass was obtained at fall harvest for miscanthus (25.4 Mg ha^{-1}) and at fall and winter harvest (23.3 and 24.0 Mg ha^{-1} , respectively) for giant reed. These results are in agreement with several experiments, showing that the highest productions are in autumn (Maughan et al., 2012; Mitchell, 2012; Larsen et al., 2014; O’Flynn et al., 2014) especially for miscanthus, which evidenced a significant decrease of biomass yield from fall to winter harvest, essentially due to the leaves loss, with an average of 7.2 leaves losses per plant, corresponding to about 2.31 Mg ha^{-1} of dry matter, confirming other results (Ragaglini, et al., 2014).

Both the perennial crops evidenced a very good potential in terms of biomass yield per hectare. The miscanthus biomass yield recorded in this study is in agreement with (Lewandoski et al., 2003), who showed that potential production of miscanthus can reach up to 25 Mg ha^{-1} in central Europe, without irrigation and with data obtained by (Giovanardi et al., 2009) in the same environment. Similarly, giant reed biomass yield obtained, is in agreement with the performances of the crop in Europe (Lewandoski et al., 2003) and comparable to a recent experiment in central Italy (26.3 Mg ha^{-1}), but where was applied annual nitrogen fertilization (Barbanti et al., 2014).

Chemical characteristics

Although ensilage and anaerobic digestion are particularly suitable for high moisture content biomass, a low dry matter content (31.4 and 34.2% for giant reed and miscanthus, respectively, at summer harvesting), associated to a very low content of soluble sugars, could affect the ensilage process, causing risk of nutrients leakage, biomass losses and mould formation (Barontini et al., 2014). Biomass moisture content

changed very differently from fall to winter harvest: in giant reed remained about stable (~50%), whilst miscanthus reduced its moisture during wintertime from 53.5% to 41.8% (data not shown), with a similar trend obtained by Monti et al. (2015). This level of dry matter, prevented a suitable silage compression to exclude air and packing; therefore, a determinate water amount was added to increase the moisture level (till to 57% for both crops) before ensilage.

Crude protein (CP) was highest at summer harvest (54.7 and 40.1 g kg⁻¹ TS) and lowest at winter harvest (28.7 and 20.6 g kg⁻¹ TS) in giant reed and miscanthus biomass, respectively. Values and trend for silages were similar, with the exception of the winter harvest, in which proteins content resulted significantly decreased in both crops (data not shown).

The C/N ratio of the biomass and silage reflected the relative N variation, since the C contents were substantially very similar for all species and harvest times (data not shown). Consequently, the ratio increased in correspondence to fall harvest, with values around 100, in biomass and silages confirming results obtained by other experiments on giant reed (Ragaglini et al., 2014; Liu et al., 2015); on the contrary, the values of both crops reach values around 200 in the silages at winter harvest.

Both perennial species, despite the very limited level of starch accumulation, showed a significant increase from summer to fall harvest time and a significant decrease from fall and winter harvest (Fig. 3). The highest starch amount was obtained at fall harvest for giant reed, (50.3 g kg⁻¹ TS), on the contrary the lowest at winter harvest for miscanthus (4.1 g kg⁻¹ TS).

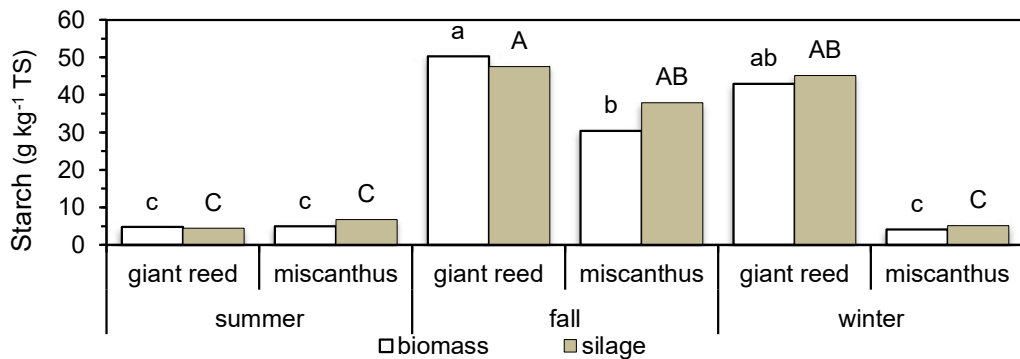


Figure 3. Starch content in fresh and post-ensilage biomass of giant reed and miscanthus at three harvest times (summer, fall and winter). Different letters, lowercase and capital letters for biomass and silage, respectively, indicate statistically different means (SNK test; $P \leq 0.05$).

Giant reed exhibited the lowest and highest SC biomass content at summer and fall harvest respectively (39.4 and 80.3 g kg⁻¹ TS) and an intermediate value at winter harvest (55.3 g kg⁻¹ TS). As temperate C3 grass, the main reserve carbohydrates are fructans (Pollock & Cairns, 1991) which are stored temporary in the stalk at the beginning of flowering (stage corresponding to fall harvest in our experiment), before uploading to the storage organs (as tubers or important rhizomes as in giant reed) at complete maturity (Maijer & Mathijssen, 1991). Conversely, miscanthus coming from another area of origin, with a different physiological functionality (C4 plant) and with

less important rhizomes with respect to giant reed, showed a SC content that increased at fall harvest ($56.1 \text{ g kg}^{-1} \text{ TS}$), remaining unchanged at winter harvest ($56.5 \text{ g kg}^{-1} \text{ TS}$) (Fig. 4).

Silages of both species showed a significant reduction (from 75 to 90%) in SC with respect to biomass content, especially in fall and winter harvest; in this last harvest the SC content showed very low values almost negligible in both species. (Fig. 4).

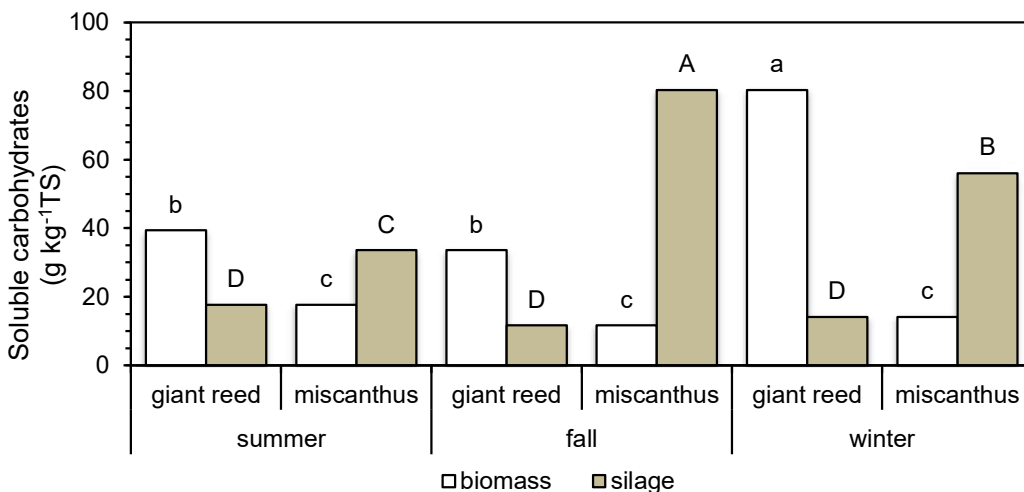


Figure 4. Water soluble carbohydrates (WSC) content in fresh and post-ensilage biomass of giant reed and miscanthus at three harvest times (summer, fall and winter). Different letters, lowercase and capital letters for biomass and silage respectively, indicate statistically different means (SNK test; $P \leq 0.05$).

Fiber components

According to ANOVA (Table 2), biomass composition clearly changed with harvesting time. Both crops showed a significant increase in lignin and cellulose content in biomass and a decrease in hemicellulose concentration in correspondence of winter harvest time, especially evident in miscanthus mainly due to a significant leaf loss when harvested in winter, confirming the results obtained by others authors (Hodgson et al., 2010; Hayes, 2013) (Table 2, Fig. 5). The increase of lignin, a widely recognized physical constrain for enzymatic hydrolysis (Pan et al., 2005), at winter harvest, could negatively affect the feedstock quality for anaerobic digestion for bio-methane production. This is confirmed by the substantial uniformity in fiber composition in silage, indicating a very limited hydrolytic activity of both crops during the ensilage process (Fig. 5). Conversely, maize seems to have a wide range of complex hydrolytic activities able to transform a part of hemicellulose in soluble sugars during ensilage (Dewar et al., 1963; Shepherd & Kung, 1996), confirming that hemicellulose is sensitive to low pH and partially hydrolysable under acidic conditions (Morrison, 1979; Jones et al., 1992; Rooke & Hatfield, 2003).

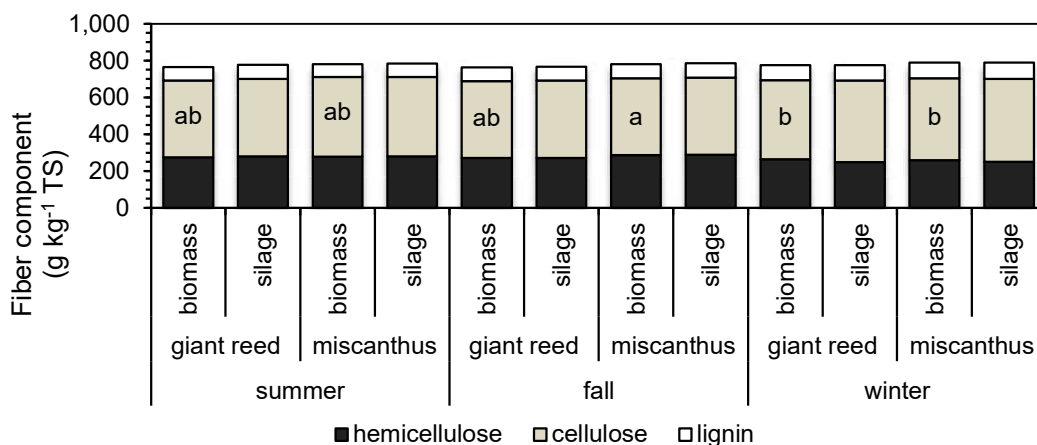


Figure 5. Fiber component in fresh and post-ensilage biomass of giant reed and miscanthus at three harvest times (summer, fall and winter). For hemicellulose in fresh biomass, different letters indicate statistically different means (*SNK test*; $P \leq 0.05$).

Silages fermentation quality

The highest levels of lactic acid were detected at fall harvest in giant reed and miscanthus (54.2 and 46.7 g kg⁻¹ TS, respectively), conversely the same acid practically disappeared in both crops silages obtained with winter harvest (Fig. 6). On the contrary, the acetic acid levels resulted very high in correspondence of winter harvest, in particular in miscanthus with a concentration (28.3 g kg⁻¹ TS) above the 20 g kg⁻¹ TS, considered a maximum threshold of a silage fermented adequately (Ferreira, 2001) (Fig. 6). Butyric acid content, that in silages with proper fermentation must show values lower than 1 g kg⁻¹ TS (Ferreira, 2001), in miscanthus ever resulted at very high concentration with values between 7.3 and 8.9 g kg⁻¹ TS, and in winter harvest the same acid resulted increased significantly also in giant reed (8.4 g kg⁻¹ TS).

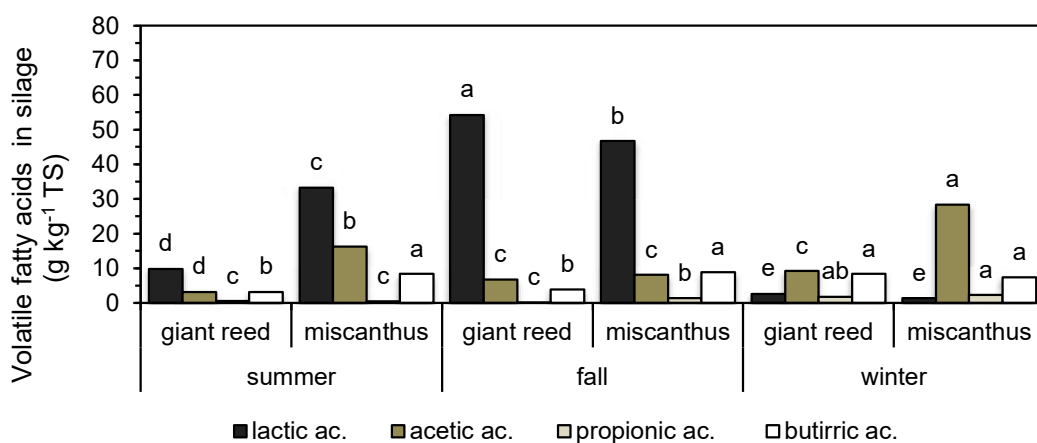


Figure 6. Volatile fatty acids (VFA) content in silage of giant reed and miscanthus at three harvest times (summer, fall and winter). For each VFA, different letters indicate statistically different means (*SNK test*; $P \leq 0.05$).

The production of ammonia nitrogen ($\text{NH}_3\text{-N}$) in silages of good quality has to be lower than 100 gN kg^{-1} of the total nitrogen (Ferreira, 2001) and values very close to the above limit were found in both crop silages harvested in fall. Conversely, the same ammonia nitrogen increased significantly in silages with summer harvest and especially with winter harvest, with values of 626.8 and 781.6 gN kg^{-1} in giant reed and miscanthus, respectively (Fig. 7). Probably the miscanthus ensilages in the winter harvest was negatively affected by the very high dry matter content (581.7 g kg^{-1}) which determined also the highest silage pH value (5.9) of the trial (data not shown). On the contrary, both silages crops harvested at fall, reached final values of pH below 4, creating favorable conditions for development of lactic acid bacteria responsible for a successful ensilage process.

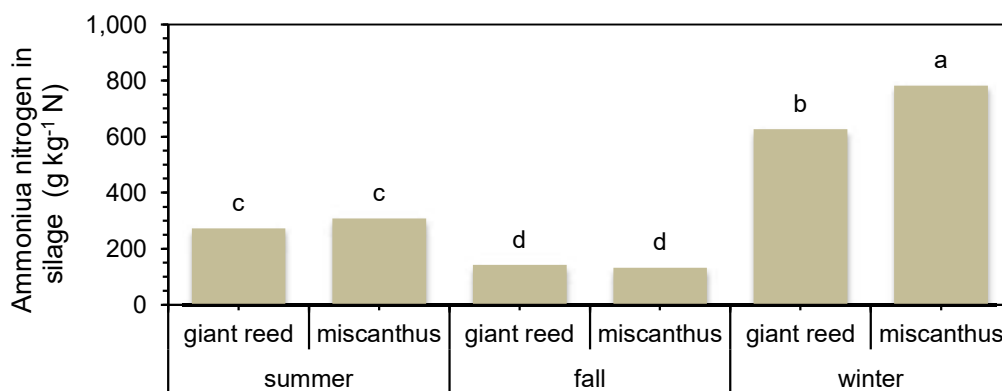


Figure 7. Ammonia nitrogen (N-NH_3) content in silage of giant reed and miscanthus at three harvest times (summer, fall and winter). Different letters indicate statistically different means (*SNK test*; $P \leq 0.05$).

Methane production (BMP and yield per hectare)

BMP tests were considered concluded when the cumulative biogas curve was addressed toward the plateau phase, precisely when daily methane production rate lowered below than $1.0 \text{ NmL CH}_4 \text{ g}^{-1} \text{ VS day}^{-1}$. This happened after 36 days of digestion for all the comparative thesis and cumulative methane productions (BMP) were calculated at that moment.

Giant reed showed the highest BMP when harvested at the fall harvest ($169.7 \text{ NmL CH}_4 \text{ g}^{-1} \text{ VS}$), conversely miscanthus at the first cut ($171.4 \text{ NmL CH}_4 \text{ g}^{-1} \text{ VS}$), and both results were significantly higher than those obtained from the other harvesting periods (Table 4). The significantly lower BMPs were found at the winter harvest, both for giant reed and miscanthus (116.6 and $113.6 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}$, respectively).

The effect on BMP of different harvesting period is largely discussed in literature. Giant reed harvested in five different periods in Mediterranean climate and submitted directly to anaerobic tests showed a BMP decrease from 332.9 to $258.3 \text{ NmL CH}_4 \text{ g}^{-1} \text{ VS}$ from June to September harvest time (Ragaglini et al., 2014). Giant reed harvested in October period showed a BMP of $150.8 \text{ NmL CH}_4 \text{ g}^{-1} \text{ VS}$.

Table 4. Biochemical methane potential (BMP), methane and energy yield of perennial crops harvested at three different times. Results are means \pm *Standard Error*

Harvest time	Crop	BMP (mL CH ₄ g ⁻¹ VS)	Bio-methane yield (m ³ CH ₄ ha ⁻¹)
Summer	G	148.1 \pm 2.8	2,676.0 \pm 263.1
	M	171.4 \pm 4.0	3,083.4 \pm 526.7
Fall	G	169.7 \pm 3.9	3,795.8 \pm 473.7
	M	159.6 \pm 1.6	3,959.9 \pm 381.3
Winter	G	116.6 \pm 3.8	2,686.5 \pm 514.7
	M	120.5 \pm 5.5	2,645.5 \pm 189.1

G = giant reed; M = miscanthus.

Yang & Li (2014) found BMPs from giant reed similar to those obtained in this study (130–150 mL CH₄ g⁻¹ VS), however operating with fresh plants and adopting a I:S ratio (1:2) different to that adopted in this study (2:1).

Miscanthus harvested after the winter showed BMP as low as 84 NmL CH₄ g⁻¹ VS (Menardo et al., 2012); conversely BMP obtained by miscanthus harvested before the winter and ensiled, showed values higher than 200 NmL CH₄ g⁻¹ VS (Mayer et al., 2014), still slightly higher than that measured in this study (171.4 NmL CH₄ g⁻¹ VS). Even higher values of BMP (345–374 mL CH₄ g⁻¹ VS) can be reached when miscanthus is pre-treated before the anaerobic digestion with steam-explosion, as confirmed by (Menardo et al., 2012).

It is conceivable that the BMP decline with harvesting season (from summer to fall-winter) was determined by an higher content in slowly digestible or un-digestible fibre (lignin, in particular) and by major problems that could have occurred during the silage, especially in relation to changes in VFA composition. High concentration of propionic and butyric acid could have an effect in reducing methane potential of giant reed and miscanthus. As the characteristics of the ensiled substrates are concerned, the highest concentration of propionic acid was found in winter harvest, amounting to 1.8–2.3 g kg⁻¹, respectively for giant reed and miscanthus. Moreover, in this study at the same winter harvest time, butyric acid in ensiled substrates was considerably higher than propionic, resulting 8.4 and 7.3 mg kg⁻¹. These values could have contribute to reach in the system values at which the activity of acidogenic bacteria is repressed, resulting in VFA accumulation, methanogenic bacteria repression and, consequently, methane production reduction, until a complete cessation (Wang et al., 2009).

The accumulation of longer chain acids (mainly propionic and butyric acids) within the system could be related to a low I:S ratio of VS. The acetate produced in first steps of the digestion at lower I:S ratio could inhibit methanogens activity and consequently an increase of I:S ratio could improve the ultimate methane yield of substrate (Raposo et al., 2011; Dechruga et al., 2013).

The combination between biomass production per hectare and BMPs lead to the highest methane productions and energy yield at the second harvest (fall), both for giant reed and miscanthus (3795.8–3959.9 m³ CH₄ ha⁻¹, respectively). It is noticeable to observe that the highest methane production was reached by miscanthus at the fall harvest, despite the BMP lower than giant reed, confirming the relevance of biomass

yield; several authors (Amon et al., 2007; Kreuger et al., 2011; Ragaglini et al., 2014) previously described similar results. Moreover, very similar results were obtained for miscanthus to that observed by Wahid et al. (2015), who concluded that the optimal harvesting period for miscanthus was between September-October ($3,824 \text{ m}^3 \text{ CH}_4 \text{ ha}^{-1}$), corresponding to the fall harvest time of this study. Conversely, methane yields per hectare obtained by giant reed were slightly lower than those reported by other authors, who, however, used fresh or dried plants (Ragaglini et al., 2014; Yang & Li, 2014) or chemical-thermal pre-treated plants (Girolamo et al., 2013). However, the methane production per hectare obtained from giant reed and miscanthus, although of some interest, was about 70%, as mean, that of maize, as reported by Baldini et al. (2017) in a similar experiment carried out in the same location.

Digestate characterization for agricultural use

Digested effluents from lab-scale batch tests, in consequence of the higher I:S rate adopted, could have very similar characteristics to effluents produced during the starting phase of a real scale digester, when substrates are loaded with a low organic loading rate, and a characterization of such effluents could be useful for assessing possible critical factors for their use in agriculture.

Digestate obtained from giant reed had a lower TS content than that obtained from miscanthus, respectively ranging from 54.0 to 49.3 g kg^{-1} and 62.9 to 56.8 g kg^{-1} (Table 5). TS content was slightly higher in summer harvest than in other harvesting periods, both for giant reed and miscanthus (Table 5). An opposite tendency was observed for VS content of digestate, which was highest at summer harvest (780 g kg^{-1} TS), and the lowest at winter harvest (630 g kg^{-1} TS) in giant reed.

Table 5. Some parameters and heavy metals content of digestate of giant reed and miscanthus harvested at three different times (summer, fall, winter)

Traits	Unit	Giant summer	Misc. summer	Giant fall	Misc. fall	Giant winter	Misc. winter
TS	g kg^{-1}	54.0	62.9	51.4	60.3	49.3	56.8
VS	g kg^{-1} TS	780.0	734.0	779.0	768.0	630.0	653.0
N	g kg^{-1} TS	21.4	20.6	28.4	25.4	30.9	22.6
C/N	-	16.0	16.0	12.6	15.2	13.1	16.9
Zn	mg kg^{-1} TS	664.3	546.7	752.0	704.9	886.7	607.4
Cu	mg kg^{-1} TS	194.6	171.3	222.4	235.5	356.2	223.5
Cr	mg kg^{-1} TS	22.5	19.5	24.5	25.0	30.7	22.7
Ni	mg kg^{-1} TS	16.8	13.6	16.1	16.2	22.6	17.4
Pb	mg kg^{-1} TS	1.9	1.7	1.6	2.4	3.8	1.9
Cd	mg kg^{-1} TS	0.8	0.6	0.9	0.8	1.0	0.7

The total N content of digestate was between 20.6 g kg^{-1} TS (miscanthus at summer harvest) and 30.9 g kg^{-1} TS (giant reed at winter harvest) (Table 5); in each harvesting time, giant reed digestate had an higher N content than miscanthus, with a general slight N increase from spring to winter harvesting. C/N ratio varied between 12.6 and 16.9 (for giant reed at summer and miscanthus at winter harvest, respectively), with a general trend lower in giant reed than in miscanthus digestate, due to the higher fiber content of this latter species, also. C/N ratio during anaerobic digestion is expected to decrease, due to the C volatilization as CO_2 and CH_4 , and the final C/N ratio of digestate normally is

stabilized on values lower than 20 (Li et al., 2011; Zeshan et al., 2012), confirming our results.

Digestate consisted of crops and inoculum as feedstock contained different amounts of microelements and heavy metals, which are important elements for the plants but also potentially dangerous for the soil (Moller & Muller, 2012). The most represented elements in digestate were Zn, Cu, Cr, Ni, Pb and Cd (Table 5), the firsts (Zn and Cu) contained as hundreds, the seconds (Cr and Ni) as dozens and the lasts (Pb and Cd) contained from 0.6 to 3.8 mg kg⁻¹ TS.

As nitrogen contained in digestate is concerned, a number of different approaches are adopted in EU member states for the calculation of 170 kg ha⁻¹ year⁻¹ limit and for the efficiency values adopted for land application of digestate as fertilizer. In this study, accounting the whole N of digestate to comply with the maximum limit of nitrogen allowable in nitrate vulnerable zones (170 kg ha⁻¹ year⁻¹), the highest distributable volume of digestate was ranging between 111.0 m³ ha⁻¹ year⁻¹ (miscanthus at fall harvest) and 147.1 m³ ha⁻¹ year⁻¹ (giant reed at summer harvest) (Table 6). Thus, in relation of the digestate distributable volumes, the maximum annual input of heavy metals in soil was calculated (Table 6).

Table 6. Digestate volume and amount of heavy metals distributable in the soil, utilizing digestate after AD of giant reed and miscanthus silage, harvested at three different harvest times (summer, fall, winter)

Traits	Unit	Giant summer	Misc. summer	Giant fall	Misc. fall	Giant winter	Misc. winter
Volume	m ³ ha ⁻¹	147.1	131.2	116.5	111.0	111.6	132.4
Zn	kg ha ⁻¹	5.28	4.51	4.50	4.72	4.88	4.57
Cu	kg ha ⁻¹	1.55	1.41	1.33	1.58	1.96	1.68
Cr	kg ha ⁻¹	0.18	0.16	0.15	0.17	0.17	0.17
Ni	kg ha ⁻¹	0.13	0.11	0.09	0.11	0.12	0.13
Pb	kg ha ⁻¹	0.015	0.014	0.010	0.016	0.020	0.014
Cd	kg ha ⁻¹	0.006	0.005	0.005	0.005	0.006	0.005

Many European Countries, indeed, have specific rules and limits for heavy metals in digestate used as fertilizer (Al Saedi et al., 2013). Conversely, the only legal limits in force in Italy are those stated in the Legislative Decree (D.Lgs. 99/92), which accomplish to the European Directive on the use of sewage sludge in agriculture (86/278/EEC). From Table 7, it is possible to compare the heavy metals concentration calculated by this study to heavy metal limits of Italy and some European Countries. It was evident that heavy metals content were largely below the limits and the only cases of threshold overcoming was for Cu and Zn, if the most compelling rules of some Northern European Countries are considered (the Netherlands, Sweden and United Kingdom). The same Cu and Zn contents would have overcome the Austria limits, which are calculated in term of heavy metals distributed over a 2 years period; in this last case, the limit overcoming was calculated also for Cd.

Table 7. Several countries limits for heavy metals in digestate used as fertilizer

Countries	Unit	Zn	Cu	Cr	Ni	Pb	Cd
Austria	g ha ⁻¹ in two years	3,000	700	600	400	600	10
Denmark	mg kg ⁻¹ TS	4,000	1,000	100	30	120	0.8
France	mg kg ⁻¹ TS	3,000	1,000	3,000	200	800	20
Germany	mg kg ⁻¹ TS	2,500	1,000	900	200	900	10
Netherland	mg kg ⁻¹ TS	300	75	75	30	100	1.25
Sweden	mg kg ⁻¹ TS	800	600	100	50	100	1.0
United Kingdom	mg kg ⁻¹ TS	400	200	100	50	200	1.5
Italy ^a	mg kg ⁻¹ TS	2,500	1,000	Nd	300	750	20
From this study	mg kg ⁻¹ TS	886.7	356.2	30.7	22.6	3.8	1.0
(max values)	g ha ⁻¹ in two years	14,087.8	5,879.0	367.5	302.5	41.8	15.0

^a – values referred to the use of sewage sludge in agriculture (D.Lgs. 99/92).

CONCLUSIONS

Both giant reed and miscanthus have demonstrated an excellent biomass aboveground potential in the environment under study despite the low-input cultivation technique adopted, with respect to maize. In particular, autumnal harvesting seems to be the most appropriate as it combines the highest biomass, bio-methane and energy yield, also allowing a good performance of the silage process, which takes place naturally, without the need for additives, providing a good quality silage.

The low attained BMP showed the bottlenecks of the methanisation of ensiled giant-reed and miscanthus with respect to maize, mainly due a general recalcitrance of lignocellulosic biomass. In this case, a feasible solution could be a suitable biomass pre-treatment able to weaken the lignocellulosic bonds, to increase the amount of water-soluble carbohydrates and consequently to enhance methane production.

The digestate obtained in this experiment, comparable to that of starting phases of a real scale digester, exceeded the legal limits allowed of Cu, Zn and Cd content if the rules of some countries (i.e. The Netherland, United Kingdom and Austria) are considered. Therefore, in countries like Italy, which have not yet specific rules, it should be a good practice to establish precise limits to heavy metals – besides to N – for calculating the maximum amount of digestate disposable in the vulnerable areas.

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REFERENCES

- Al Saedi, T., Drogg, B., Fuchs, W., Rutz, D. & Janssen, R. 2013. Biogas digestate quality and utilization. In: Wellinger, A., Murphy, J.D. & Baxter, D. (ed.): *The Biogas Handbook: Science, Production and Applications*. WP Woodhead Publishing Limited, 267–298.
- Amon, T., Amon, B., Kryvoruchko, V., Machmuller, A., Hopfner-Sixt, K., Bodiroza, V., Hrbek, R., Friedel, J., Potsch, E., Wagentristl, H., Schreiner, M. & Zollitsch, W. 2007. Methane production through anaerobic digestion of various energy crops grown in sustainable crop rotations. *Bioresource Technol.* **98**, 3204–3212.

- Angelini, L.G., Ceccarini, L. & Bonari, E. 2005. Biomass yield and energy balance of giant reed (*Arundo donax* L.) cropped in central Italy as related to different management practices. *Eur. J. Agron.* **22**, 375–89.
- Angelini, L.G., Ceccarini, L., Nasso, N. & Bonari, E. 2009. Comparison of *Arundo donax* L. and *Miscanthus x giganteus* in a long-term field experiment in Central Italy: analysis of productive characteristics and energy balance. *Biomass Bioenerg.* **33**, 635–643.
- ARPA FVG–OSMER 2016. Schede climatiche del Friuli Venezia Giulia. 311. <http://www.clima.fvg.it>. Accessed 05 January 2017 (in Italian).
- Arundale, R., Dohleman, F., Heaton, E., Mcgrath, J., Voigt, T. & Long, S. 2014. Yields of *Miscanthus x giganteus* and *Panicum virgatum* decline with stand age in the Midwestern USA. *GCB Bioenergy* **6**(1), 1–13.
- Baldini, M., Da Borso, F., Ferfua, C., Zuliani, F. & Danuso, F. 2017. Ensilage suitability and bio-methane yield of *Arundo donax* and *Miscanthus x giganteus*. *Ind. Crop. Prod.* **95**, 264–275.
- Barbanti, L., Girolamo, G.D., Grigatti, M., Bertin, L. & Ciavatta, C. 2014. Anaerobic digestion of annual and multi-annual biomass crops. *Ind. Crop. Prod.* **56**, 137–144.
- Barontini, M., Crognale, S., Scarfone, A., Gallo, P., Gallucci, F., Petruccioli, M., Pesciaroli, L. & Pari, L. 2014. Airborne fungi in biofuel wood chip storage sites. *Int. Biodeter. Biodegr.* **90**, 17–22.
- Baxter, X.C., Darvell, L.I., Jones, J.M., Barraclough, T., Yates, N.E. & Shield, I. 2014. *Miscanthus* combustion properties and variations with *miscanthus* agronomy. *Fuel* **117**, 851–869.
- Beale, C.V. & Long, S.P. 1997. Seasonal dynamics of nutrient accumulation and partitioning in the perennial C4-grasses *Miscanthus x giganteus* and *Spartina cynosuroides*. *Biomass Bioenerg.* **12**(6), 419–428.
- Cadoux, S., Ferchaud, F., Demay, C., Zard, H.B., Machet, J.M., Fourdinier, E., Preudhomme, M., Chabbert, B., Gosse, G. & Mary, B. 2014. Implications of productivity and nutrient requirements on greenhouse gas balance of annual and perennial bioenergy crops. *GCB Bioenergy* **6**, 425–438.
- Carrosio, G. 2013. Energy production from biogas in the Italian countryside: policies and organizational models. *Energ. Policy* **63**, 3–9.
- Dechruga, S., Kantachote, D. & Chaiprapat, S. 2013. Effects of inoculum to substrate ratio, substrate mix ratio and inoculum source on batch co-digestion of grass and pig manure. *Bioresour. Technol.* **146**, 101–108.
- Dewar, W., McDonald, P. & Whittenbury, R. 1963. The hydrolysis of grass hemicelluloses during ensilage. *J. Sci. Food Agr.* **14**, 411–417.
- Dragoni, F., Ragolini, G., Corneli, E., Nasso, N. o di Nasso, Tozzini, C., Cattani, S. & Bonari, E. 2015. Giant reed (*Arundo donax* L.) for biogas production: land use saving and nitrogen utilisation efficiency compared with arable crops. *Ital. J. Agron.* **10**, 664.
- FAO. 2008. The State of Food and Agriculture. BIOFUELS: prospects, risks and opportunities. FAO Agriculture Series No. **38**. Rome.
- Fazio, S. & Monti, A. 2011. Life cycle assessment of different bioenergy production systems including perennial and annual crops. *Biomass Bioenerg.* **35**, 4868–4878.
- Ferreira, J.J. 2001. Estágio de maturação ideal para ensilagem do milho e do sorgo. In Cruz, J.C., Pereira Filho, I.A., Rodrigues, J.A.S. et al. (ed.): *Produção e utilização de silagem de milho e sorgo*. Sete Lagoas: Embrapa Milho e Sorgo, pp. 405–428.
- Giovanardi, R., Castelluccio, M.D., Rosso, S., Tassan-Mazzocco, G. & Picco, D. 2009. La coltivazione del Miscanto in Friuli Venezia Giulia: aspetti agronomici, energetici e ambientali. *Notiziario ERSA* **3**, 42–48 (in Italian).
- Goering, H.K. & Van Soest, P.J. 1970. Forage Fiber Analysis (apparatus, reagents, procedures and some applications). *USDA Agricultural Handbook* No. **379**.

- Hastings, A., Clifton-Brown, J., Wattenbach, M., Stampfl, P., Mitchell, C. & Smith, P. 2008. Potential of Miscanthus grasses to provide energy and hence reduce greenhouse gas emissions. *Agron. Sustain. Dev.* **28**, 465–472.
- Hayes, D.J.M. 2013. Mass and compositional changes relevant to biorefining, in Miscanthus x giganteus plants over the harvest window. *Bioresource Technol.* **142**, 591–602.
- Heaton, E.A., Dohleman, F.G. & Long, S.P. 2009. Seasonal nitrogen dynamics of Miscanthus x giganteus and Panicum virgatum. *GCB Bioenergy* **1**, 297–307.
- Heaton, E.A., Long, S.P., Voigt, T.B., Jones, M.B. & Clifton-Brown, J. 2004. Miscanthus for renewable energy generation: European Union experience and projections for Illinois. *Mitig. Adapt. Strat. GL* **9**, 433–51.
- Herrmann, C., Heiermann, M. & Idler, C. 2011. Effects of ensiling, silage additives and storage period on methane formation of biogas crops. *Bioresource Technol.* **102**, 5153–5161.
- Hoagland, K.D., Ruark, M.D., Renz, M.J. & Jackson, R.D. 2013. Agricultural management of switchgrass for fuel quality and thermal energy yield on highly erodible land in the driftless area of southwest Wisconsin. *Bioenerg. Res.* **6**, 1012–1021.
- Hodgson, E.M., Lister, S.J., Bridgwater, A.V., Clifton-Brown, J. & Donnison, I.S. 2010. Genotypic and environmentally derived variation in the cell wall composition of Miscanthus in relation to its use as a biomass feedstock. *Biomass Bioenerg.* **34**, 652–660.
- Jones, B., Hatfield, R. & Muck, R. 1992. Effect of fermentation and bacterial inoculation on lucerne cell walls. *J. Sci. Food Agr.* **60**, 147–153.
- Klimiuk, E., Pokój, T., Budzyński, W. & Dubis, B. 2010. Theoretical and observed biogas production from plant biomass of different fiber contents. *Bioresource Technol.* **101**, 9527–9535.
- Kludze, H., Deen, B. & Dutta, A. 2013. Impact of agronomic treatments on fuel characteristics of herbaceous biomass for combustion. *Fuel Process. Technol.* **109**, 96–102.
- Kreuger, E., Prade, T., Escobar, F., Svensson, S., Englund, J. & Bjornsson, L. 2011. Anaerobic digestion of industrial hemp—Effect of harvest time on methane energy yield per hectare. *Biomass Bioenerg.* **35**, 893–900.
- Larsen, S.U., Jørgensen, U., Kjeldsen, J.B. & Lærke, P.E. 2014. Long-term Miscanthus yields influenced by location, genotype, row distance, fertilization and harvest season. *Bioenerg. Res.* **7**, 620–635.
- Lewandowski, I., Scurlock, J.M., Lindvall, E. & Christou, M. 2003. The development and current status of perennial rhizomatous grasses as energy crops in the US and Europe. *Biomass Bioenerg.* **25**, 335–361.
- Li, Y., Park, S.Y. & Zhu, J. 2011. Solid-state anaerobic digestion for methane production from organic waste. *Renew. Sust. Energ. Rev.* **15**, 821–826.
- Liu, S., Ge, X., Liew, L.N., Liu, Z. & Li, Y. 2015. Effect of urea addition on giant reed ensilage and subsequent methane production by anaerobic digestion. *Bioresource Technol.* **192**, 682–688.
- Maijer, W. & Mathijssen, E.W.J.M. 1991. The relation between flower initiation and sink strength of stems and tubers of Jerusalem artichoke. *Neth. J. Agr. Sci.* **39**, 123–135.
- Mantione, M., D'Agosta, G.M., Copani, V., Patane, C. & Cosentino, S.L. 2009. Biomass yield and energy balance of three perennial crops for energy use in the semi-arid Mediterranean environment. *Field Crop. Res.* **114**, 204–213.
- Massé, D., Gilbert, Y., Savoie, P., Bélanger, G., Parent, G. & Babineau, D. 2010. Methane yield from switchgrass harvested at different stages of development in Eastern Canada. *Bioresour. Technol.* **101**, 9536–9541.
- Maughan, M., Bollero, G.N., Lee, D.K., Rarmody, R., Bonos, S., Cortese, L., Murphy, J., Gaussoin, R., Sousek, M., Williams, D., Williams, L., Miguez, F. & Voigt, T. 2012. Miscanthus × giganteus productivity: the effects of management in different environments. *GCB Bioenergy* **4**, 253–265.

- Mayer, F., Gerin, P.A., Noo, A., Lemaigre, S., Stilmant, D., Schmit, T., Leclech, N., Ruelle, L., Gennen, J. & Von Francken-Welz, H. 2014. Assessment of energy crop salter-native to maize for biogas production in the Greater Region. *Bioresource Technol.* **166**, 358–67.
- Menardo, S., Airoldi, G. & Balsari, P. 2012. The effect of particle size and thermal pre-treatment on the methane yield of four agricultural by-products. *Bioresource Technol.* **104**, 708–714.
- McCleary, B.V., Gibson, T.S., Mugford, D. C. 1997. Measurement of total starch in cereal products by amyloglucosidase - α -amylase method: Collaborative study. *J. AOAC Int.* **80**, 571–579.
- Mitchell, M.S.R. 2012. Switchgrass harvest and management. In Monti, A. (Ed.), *Switchgrass*, I. Springer-Verlag, London, UK, **1**, pp. 113–127.
- Moller, K. & Müller, T. 2012. Effects of anaerobic digestion on digestate nutrient availability and crop growth: A review. *Eng. Life Sci.* **12**(3), 242–257.
- Monlau, F., Barakat, A., Trably, E., Dumas, C., Steyer, J. & Carrere, H. 2013. Lignocellulosic materials into biohydrogen and biomethane: impact of structural features and pretreatment. *Crit. Rev. Env. Sci. Tec.* **43**, 260–322.
- Monti, A., Zanetti, F., Scordia, D., Testa, G. & Cosentino, S.L. 2015. What to harvest when? Autumn, winter, annual and biennial harvesting of giant reed, miscanthus and switchgrass in northern and southern Mediterranean area. *Ind. Crop. Prod.* **75**, 129–134 (Part B).
- Morrison, I. 1979. Changes in the cell wall components of laboratory silages and the effects of various additives on these changes. *J. Agr. Sci.* **93**, 581–586.
- Neureiter, M., Dos Santos, J., Lopez, C., Pichler, H., Kirchmayr, R. & Braun, R. 2005. Effect of silage preparation on methane yields from whole crop maize silages. *Proceedings of the 4th International Symposium on Anaerobic Digestion of Solid Waste*. Copenhagen, Denmark, **1**, pp. 109–115.
- O'Flynn, M.G., Finnan, J.M., Curley, E.M. & McDonnell, K.P. 2014. Reducing crop damage and yield loss in late harvests of *Miscanthus × giganteus*. *Soil Till. Res.* **140**, 8–19.
- Pan, X., Xie, D., Gilkes, N., Gregg, D.J. & Saddler, J.N. 2005. Strategies to enhance the enzymatic hydrolysis of pretreated softwood with high residual lignin content. *Appl. Biochem. Biotech.* **121**, 1069–1079.
- Pollock, C.J. & Cairns, A.J. 1991. Fructan metabolism in grasses and cereals. *Annu. Rev. Plant. Phys.* **42**, 77–101.
- Ragaglini, G., Dragoni, F., Simone, M. & Bonari, E. 2014. Suitability of giant reed (*Arundo donax* L.) for anaerobic digestion: effect of harvest time and frequency on the biomethane yield potential. *Bioresource Technol.* **152**, 107–115.
- Raposo, F., Fernández-Cegri, V., De la Rubia, M.A., Borja, R., Béline, F., Cavinato, C., Demirer, G., Fernández, B., Fernández-Polanco, M., Frigon, J., Ganesh, R., Kaparaju, P., Koubova, J., Méndez, R., Menin, G., Peene, A., Scherer, P., Torrijos, M., Uellendahl, H., Wierinck, I. & de Wilde, V. 2011. Biochemical methane potential (BMP) of solid organic substrates: evaluation of anaerobic biodegradability using data from an international interlaboratory study. *J. Chem. Technol. Biot.* **86**, 1088–1098.
- Richardson, B. 2012. From a fossil-fuel to a biobased economy: The politics of industrial biotechnology. *Environ. Plann. C* **30**, 282–296.
- Rooke, J.A. & Hatfield, R.D. 2003. Biochemistry of Ensiling. USDA-ARS/UNL Faculty, Paper 1399. <http://digitalcommons.unl.edu/usdaarsfacpub/1399>. Accessed 01.06.16.
- Sheperd, A. & Kung, L. 1996. Effects of an enzyme additive on composition of corn silage ensiled at various stages of maturity. *J. Dairy Sci.* **79**, 1767–1773.
- USEPA (U.S. Environmental Protection Agency). 2007. Nanotechnology white paper - external review draft. EPA 100/B-07/001.
Available from: <https://www.epa.gov/osa/nanotechnology-white-paper>

- Vervaeren, H., Hostyn, K., Ghekiere, G. & Willems, B. 2010. Biological ensilage additives as pretreatment for maize to increase the biogas production. *Renew. Energ.* **35**, 2089–2093.
- Wahid, R., Nielsen, S., Hernandez, V.M., Ward, A., Rene Gislum, R., Jørgensen, U. & Møller, H. 2015. Methane production potential from *Miscanthus* sp. Effect of harvesting time, genotypes and plant fractions. *Biosyst. Eng.* **133**, 71–80.
- Wang, Y., Zhang, Y., Wang, J. & Meng, L. 2009. Effects of volatile fatty acid concentrations on methane yield and methanogenic bacteria. *Biomass Bioenerg.* **33**, 848–853.
- Weiland, P. 2006. State of the art of solid-state digestion-recent developments. In Rohstoffe, F.N. (ed.): *Solid-State Digestion-State of the Art and Further R&D Requirements*. Gulzower Fachgespräche, **24**, pp. 22–38.
- Yahaya, M.S., Kimura, A., Harai, J., Nguyen, H.V., Kawai, M., Takahashi, J. & Matsuoka, S. 2001. Effect of length of ensiling on silo degradation and digestibility of structural carbohydrates of Lucerne and orchardgrass. *Anim. Feed Sci. Tech.* **92**, 141–148.
- Yang, L. & Li, Y. 2014. Anaerobic digestion of giant reed for methane production. *Bioresource Technol.* **171**, 233–239.
- Zeshan, Karthikeyan, O.P. & Visvanathan, C. 2012. Effect of C/N ratio and ammonia-N accumulation in a pilot-scale thermophilic dry anaerobic digester. *Bioresource Technol.* **113**, 294–302.